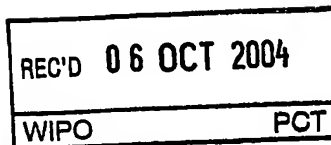




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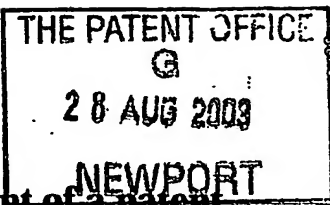
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*R. Mahoney*

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Dated 9 September 2004



1/77

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28AUG03 FB33248-6 B02804  
P01/7700 0.00-0320122.5

1. Your reference

P342351/CPA/MCM

2. Patent application number

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28 AUG 2003

0320122.5

3. Full name, address and postcode of the or of each applicant (underline all surnames)

Albachem Limited  
Elvingston Science Centre  
by Gladsmuir  
East Lothian  
EH53 1EH

16 Charlotte Square  
Edinburgh  
EH2 4DF

086417970012

Patents ADP number (if you know it)

If the applicant is a corporate body, give the country/state of its incorporation

United Kingdom

4. Title of the invention

"Ligation Method"

5. Name of your agent (if you have one)

Murgitroyd & Company

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

Scotland House  
165-169 Scotland Street  
Glasgow  
G5 8PL

Patents ADP number (if you know it)

1198013

00001198015

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and (if you know it) the or each application number

Country

Priority application number  
(if you know it)

Date of filing  
(day / month / year)

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application

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8. Is a statement of inventorship and of right to grant of a patent required in support of this request? (Answer 'Yes' if:

Yes

- a) any applicant named in part 3 is not an inventor, or
- b) there is an inventor who is not named as an applicant, or
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See note (d))

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37

Claim(s)

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-

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Priority documents

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Statement of inventorship and right to grant of a patent (*Patents Form 7/77*)

Request for preliminary examination and search (*Patents Form 9/77*)

Request for substantive examination (*Patents Form 10/77*)

Any other documents  
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Murgitroyd & Company

Date

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Murgitroyd & Company

12. Name and daytime telephone number of person to contact in the United Kingdom

Malcolm C Main

0141 307 8400

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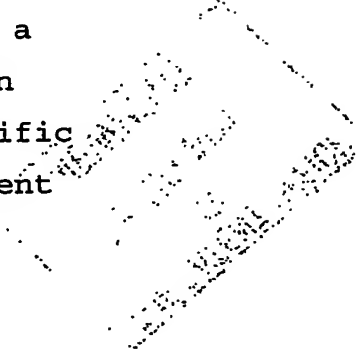
## Ligation Method

### Field of the Invention

This application relates to a method of ligating two or more molecules, for example, small organic molecules, labels, peptides etc. In particular it relates to a method of ligating a peptide, such as ligation of a synthetic peptide to a recombinant peptide.

### Background to the Invention

Protein engineering methodologies have proven to be invaluable for generating protein based tools for application in basic research, diagnostics, drug discovery and as protein therapeutics. The ability to manipulate the primary structure of a protein in a controlled manner opens up many new possibilities in the biological and medical sciences. As a consequence, there is a concerted effort on developing methodologies for the site-specific modification of proteins and their subsequent application.



1  
2 The two main approaches to generating proteins are  
3 through recombinant methods or chemical synthesis.  
4 To date, the two methods have proved to be  
5 complementary; recombinant methodologies enable  
6 proteins of any size to be generated but in general  
7 they are restricted to the assembly of the  
8 proteinogenic amino acids. Thus, in general, the  
9 introduction of labels and probes into recombinant  
10 proteins has to be implemented post-translationally  
11 and does not allow modifications to the protein  
12 backbone.

13  
14 The most common methods for labelling a recombinant  
15 protein use an amino or a thiol reactive version of  
16 the label that will covalently react with a lysine  
17 side chain /  $N^{\alpha}$  amino group or a cysteine side chain  
18 within the protein respectively. For such labelling  
19 methods to be site-specific, an appropriate  
20 derivative of the protein must be engineered to  
21 contain a unique reactive functionality at the  
22 position to be modified. This requires all the other  
23 naturally occurring reactive functionalities within  
24 the primary sequence to be removed through amino  
25 acid mutagenesis. In the case of protein amino  
26 functionalities, this is essentially impossible due  
27 to the abundance of lysine residues and the presence  
28 of the amino functionality at the N-terminus.  
29 Likewise, for cysteine this process is laborious and  
30 is often detrimental to the function of the protein.  
31

1 The production of proteins having site-specific  
2 modifications and/or labels is more readily  
3 achievable using chemical synthesis methods. The  
4 chemical synthesis of proteins, however, enables  
5 multiple modifications to be incorporated into both  
6 side-chain and backbone moieties of the protein in a  
7 site-specific manner, but, in general, the maximum  
8 size of sequence that can be synthesised and  
9 isolated is circa 50 - 100 amino acids.

10

#### 11 Protein Ligation

12 A further approach to the generation of proteins is  
13 protein / peptide ligation. In this approach  
14 mutually reactive chemical functionalities  
15 (orthogonal to the chemistry of the naturally  
16 occurring amino acids i.e. which react by mutually  
17 exclusive chemistries compared to the reactions of  
18 the reactive moieties of the naturally occurring  
19 amino acids) are incorporated at the N- and C-  
20 termini of unprotected polypeptide fragments such  
21 that when they are mixed, they react in a  
22 chemoselective manner to join the two sequences  
23 together (Cotton GJ and Muir TW. Chem.Biol., 1999,  
24 6, R247-R254). The principle of chemical ligation is  
25 shown schematically in Figure 1.

26

27 A number of chemistries have been utilised for the  
28 ligation of two synthetic peptides where a diverse  
29 range of different chemical functionalities can be  
30 incorporated into the termini of polypeptides using  
31 solid phase peptide synthesis. These include the  
32 reaction between a thioacid and bromo- alkyl to

1 form a thioester (Schnolzer M and Kent SBH, *Science*,  
2 1992, 256, 221-225), reaction of an aldehyde with an  
3 N-terminal cysteine or threonine to form  
4 thiazolidine or oxazolidine respectively (Liu C-F  
5 and Tam J P. *Proc. Natl. Acad. Sci. USA*, 1994, 91,  
6 6584 - 6588), reaction between a hydrazide and an  
7 aldehyde to form a hydrazone (Gaertner HF et al, et  
8 al *Bioconj. Chem.*, 1992, 3, 262 - 268) reaction of  
9 an aminoxy group and an aldehyde to form an oxime  
10 (Rose K. *J. Am. Chem. Soc.*, 1994, 116, 30-33),  
11 reaction of azides and aryl phosphines to form an  
12 amide bond (Staudinger ligation) (Nilsson BL,  
13 Kiessling LL, and Raines RT. *Org. Lett.*, 2001, 3, 9-  
14 12, Kiick et al *Proc. Natl. Acad. Sci. USA*, 2002,  
15 99, 19-24) , and the reaction of a peptide C-  
16 terminal thioester and an N-terminal cysteine  
17 peptide to form a native amide bond (Dawson et al.  
18 *Science*, 1994, 266, 776) (Native chemical ligation  
19 US6184344, EP 0832 096 B1). This method is an  
20 extension of studies by Wieland and coworkers who  
21 showed that the reaction of ValSPh and CysOH in  
22 aqueous buffer yielded the dipeptide ValCysOH  
23 (Wieland T et al, *Liebigs Ann. Chem.*, 1953, 583,  
24 129-149).  
25  
26 Although the native chemical ligation method has  
27 proved popular, it requires an N-terminal cysteine  
28 and thus, if a cysteine is not present at the  
29 appropriate position in the protein, a cysteine  
30 needs to be introduced at the ligation site.  
31 However, the introduction of extra thiol groups into  
32 a protein sequence maybe detrimental to its

1 structure / function, especially since cysteine has  
2 a propensity to form disulfide bonds which may  
3 disrupt the folding pathway or compromise the  
4 function of the folded protein.

5  
6 As a consequence of the difficulties and problems  
7 associated with known ligation techniques, the  
8 ligation of two synthetic fragments generally only  
9 enables proteins of circa 100 - 150 amino acids to  
10 be chemically synthesised. Although larger proteins  
11 have been synthesised by ligating together more than  
12 two fragments, this has proved to be technically  
13 difficult (Camarero et al. *J. Pept. Res.*, 1998, 54,  
14 303-316, Canne LE et al, *J. Am. Chem. Soc.*, 1999,  
15 121, 8720-8727).

16

#### 17 Protein semi-synthesis

18

19 protein ligation technologies that enable both  
20 synthetic and recombinantly derived protein  
21 fragments to be joined together have been  
22 described. This enables large proteins to be  
23 constructed from combinations of synthetic and  
24 recombinant fragments allowing proteins to be site-  
25 specifically modified with both natural and  
26 unnatural entities. By utilising such so-called  
27 protein semi-synthesis, many different synthetic  
28 moieties can be site-specifically incorporated at  
29 multiple different sites within a target protein.

30

31 In order to utilise recombinant proteins in ligation  
32 strategies the recombinant fragments must contain



1 the appropriate reactive functionalities to  
2 facilitate ligation. One approach to introduce a  
3 unique reactive functionality into a recombinant  
4 protein has been through the periodate oxidation of  
5 N-terminal serine containing sequences. Such  
6 treatment converts the N-terminal serine into a  
7 glyoxyl moiety, which contains an N-terminal  
8 aldehyde. Synthetic hydrazide containing peptides  
9 have then been ligated to the N-terminus of these  
10 protein in a chemoselective manner through hydrazone  
11 bond formation with the protein N-terminal aldehyde  
12 group (Gaertner HF et al, et al Bioconj. Chem.,  
13 1992, 3, 262 - 268, Gaertner HF, et al. *J. Biol.*  
14 *Chem.*, 1994, 269, 7224-7230). Another approach has  
15 been to generate recombinant proteins with N-  
16 terminal cysteine residues. Synthetic peptides  
17 containing C-terminal thioesters have then been  
18 site-specifically attached to the N-terminus of  
19 these proteins via amide bond formation in a manner  
20 analogous to 'native chemical ligation' (Cotton GJ  
21 and Muir TW. *Chem. Biol.*, 2000, 7, 253-261). However  
22 as with the ligation of synthetic peptides using  
23 native chemical ligation techniques, the technology  
24 requires a cysteine to be introduced at the ligation  
25 site if the primary sequence does not contain one at  
26 the appropriate position.

27

## 28 Protein Splicing Techniques

29

30 Recently technologies have been developed which  
31 enable recombinant proteins containing C-terminal  
32 thioester groups to be generated. The C-terminal

1 thioester functionality provides a unique reactive  
2 chemical group within the protein that can be  
3 utilised for protein ligation. Recombinant C-  
4 terminal thioester proteins are produced by  
5 manipulating a naturally occurring biological  
6 phenomenon known as protein splicing (Paulus H. Annu  
7 *Rev Biochem* 2000, 69, 447-496). Protein splicing is  
8 a post-translational process in which a precursor  
9 protein undergoes a series of intramolecular  
10 rearrangements which result in precise removal of an  
11 internal region, referred to as an intein, and  
12 ligation of the two flanking sequences, termed  
13 exteins (Figure 2). While there are generally no  
14 sequence requirements in either of the exteins,  
15 inteins are characterised by several conserved  
16 sequence motifs and well over a hundred members of  
17 this protein domain family have now been identified.

18  
19 The first step in protein splicing involves an N→S  
20 (or N→O) acyl shift in which the N-extein unit is  
21 transferred to the sidechain SH or OH group of a  
22 conserved Cys/Ser/Thr residue, always located at the  
23 immediate N-terminus of the intein. Insights into  
24 this mechanism have led to the design of a number of  
25 mutant inteins which can only promote the first step  
26 of protein splicing (Chong et al. *Gene*. 1997, 192,  
27 271-281, (Noren et al., *Angew. Chem. Int. Ed. Engl.*,  
28 2000, 39, 450-466). Proteins expressed as in frame  
29 N-terminal fusions to one of these engineered  
30 inteins can be cleaved by thiols via an  
31 intermolecular transthioesterification reaction, to  
32 generate the recombinant protein C-terminal

1 thioester derivative (Figure 3) (Chong et al *Gene*.  
2 1997, 192, 271-281, (Noren et al., *Angew. Chem. Int.*  
3 *Ed. Engl.*, 2000, 39, 450-466) (New England Biolabs  
4 Impact System WO 00/18881, WO 0047751). Peptide  
5 sequences containing an N-terminal cysteine residue  
6 can then be specifically ligated to the C-termini of  
7 such recombinant C-terminal thioester proteins (Muir  
8 et al *Proc. Natl. Acad. Sci. USA.*, 1998, 95, 6705-  
9 6710, Evans Jr et al. *Prot. Sci.*, 1998, 7, 2256-  
10 2264) , in a procedure termed expressed protein  
11 ligation (EPL) or intein-mediated protein ligation  
12 (IPL). As with the previously described ligation  
13 techniques, such an approach requires a cysteine to  
14 be introduced at the ligation site if one is not  
15 suitably positioned with the primary protein  
16 sequence and thus is subject to the limitations and  
17 associated with the problems of these approaches,  
18 such as the potential problems associated with the  
19 introduction of an extra thiol group into the  
20 primary sequence.

21  
22 The chemoselective ligation of N-terminal cysteine  
23 containing peptides to C-terminal thioester  
24 containing peptides, be they synthetic or  
25 recombinant, is performed typically at slightly  
26 basic pH and in the presence of a thiol cofactor.  
27 The strategy also requires a cysteine to be  
28 introduced at the ligation site, if one is not  
29 suitably positioned within the primary sequence.  
30 These requirements of this ligation approach have  
31 the potential to alter the structure or function of

1 both the protein ligation product and the initial  
2 reactants.

3

#### 4 Protein labelling

5

6 Historically protein ligation means the joining  
7 together of two peptide / protein fragments but this  
8 is synonymous with protein labelling whereby the  
9 label is a peptide or derivatised peptide. Equally  
10 if a small non-peptidic synthetic molecule contains  
11 the necessary reactive chemical functionality for  
12 protein ligation, then ligation of the synthetic  
13 molecule directly to either the N- or C- termini of  
14 the protein affords site-specific labelling of the  
15 protein. Thus technologies developed for the  
16 ligation of protein fragments can also be used for  
17 the direct labelling of either the N- or C- termini  
18 of peptides or proteins in a site - specific manner  
19 irrespective of their sequence.

20

21 Recombinant proteins containing N-terminal glyoxyl  
22 functions (generated through periodate oxidation of  
23 the corresponding N-terminal serine protein) have  
24 been site-specific N-terminally labelled through  
25 reaction with hydrazide or aminoxy derivatives of  
26 the label (Geoghegan KF and Stroh JG. *Bioconj Chem.*,  
27 1992, 3, 138-146, Alouni S et al. *Eur. J. Biochem.*,  
28 1995, 227, 328 - 334). Also recombinant proteins  
29 containing N-terminal cysteine residues have been N-  
30 terminally labelled through reaction with thioester  
31 containing labels, the label being the acyl  
32 substituent of the thioester (Schuler B and Pannell

1 LK. *Bioconjug. Chem.*, 2002, 13, 1039-43) and  
2 aldehyde (Zhao et al. *Bioconj. Chem.*, 1999, 10,  
3 424-430) functionalities to form amides and  
4 thiazolidines respectively.

5  
6 Though a number of methods for ligation of proteins  
7 exist each one has its potential drawbacks. There  
8 is thus a need for novel ligation methodologies,  
9 especially those that are compatible with both  
10 synthetic and recombinant fragments, which will  
11 complement the existing technologies and add another  
12 string to the protein engineers' bow.

#### 13 14 **Summary of the Invention**

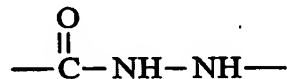
15  
16 The present inventors have overcome a number of  
17 problems associated with the prior art and have  
18 developed a new method for ligating peptide  
19 molecules which overcomes a number of the problems  
20 of the prior art.

21  
22 Accordingly, in a first aspect of the present  
23 invention, there is provided a method of producing  
24 an oligopeptide product, the method comprising the  
25 steps:

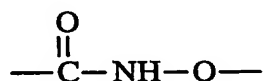
- 26 a) providing a first oligopeptide, the first
- 27 oligopeptide having a reactive moiety,
- 28 b) providing a second oligopeptide, the second
- 29 oligopeptide having a activated ester moiety
- 30 c) allowing the reactive moiety of the first
- 31 oligopeptide to react with the activated ester
- 32 moiety of the second oligopeptide to form an

oligopeptide product, in which the first and second oligopeptides are linked via a linking moiety having Formula I, Formula II or Formula III.

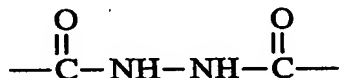
Formula I



Formula II



Formula III



In preferred embodiments, in step (c), where said oligopeptides are linked via a linking moiety having Formula II and where said activated ester moiety of step (b) is not a thioester, said activated ester is a terminal activated ester moiety.

In further preferred embodiments of the invention, said linking moieties are linked via a linking moiety having Formula I or Formula III.

Unless the context demands otherwise, the terms peptide, oligopeptide, polypeptide and protein are used interchangeably.

1 The activated ester moiety of the first oligopeptide  
2 may be any suitable activated ester moiety, such as  
3 a thioester moiety a phenolic ester moiety, an  
4 hydroxysuccinimide moiety, or an O-acylisourea  
5 moiety.

6  
7 In preferred embodiments of the invention, the  
8 activated ester moiety is a thioester moiety. Any  
9 suitable thioester peptides may be used in the  
10 present invention. In preferred embodiments, the  
11 thioester is a thioester wherein the peptide is the  
12 acyl substituent of the thioester.

13  
14 Such thioester peptides may be synthetically or  
15 recombinantly produced. The skilled person is well  
16 aware of methods known in the art for generating  
17 synthetic peptide thioesters. For example, synthetic  
18 peptide thioesters may be produced via synthesis on  
19 a resin that generates a C-terminal thioester upon  
20 HF cleavage (Hojo et al, Bull. Chem. Soc. Jpn.,  
21 1993, 66, 2700-2706). Further, the use of 'safety  
22 catch' linkers has proved to be popular for  
23 generating C-terminal thioesters through thiol  
24 induced resin cleavage of the assembled peptide  
25 (Shin Y et al, J. Am. Chem. Soc., 1999, 121, 11684-  
26 11689).

27  
28 Moreover, recently technologies have been developed  
29 which enable recombinant C-terminal thioester  
30 proteins to be generated. Recombinant C-terminal  
31 thioester proteins may be produced by manipulating a  
32 naturally occurring biological phenomenon known as

1 protein splicing. As described above, protein  
2 splicing is a post-translational process in which a  
3 precursor protein undergoes a series of  
4 intramolecular rearrangements which result in  
5 precise removal of an internal region, referred to  
6 as an intein, and ligation of the two flanking  
7 sequences, termed exteins.

8  
9 As described above, a number of mutant inteins which  
10 can only promote the first step of protein splicing  
11 have been designed (Chong et al *Gene*. 1997, 192,  
12 271-281, Noren et al., *Angew. Chem. Int. Ed. Engl.*,  
13 2000, 39, 450-466). Proteins expressed as in frame  
14 N-terminal fusions to one of these engineered  
15 inteins can be cleaved by thiols via an  
16 intermolecular transthioesterification reaction, to  
17 generate the recombinant protein C-terminal  
18 thioester derivative (Chong et al *Gene*. 1997, 192,  
19 271-281, Noren et al., *Angew. Chem. Int. Ed. Engl.*,  
20 2000, 39, 450-466) (New England Biolabs Impact  
21 System WO 00/18881, WO 0047751). Such protein  
22 thioesters may be used in the methods of the  
23 invention (See Figure 3).

24  
25 Accordingly, in a preferred aspect of the present  
26 invention, in step (b), the second oligopeptide is  
27 generated by thiol reagent induced cleavage of an  
28 intein.

29  
30 Accordingly, in a second aspect of the present  
31 invention, there is provided a method of producing  
32 an oligopeptide product, the method comprising the



1 steps:

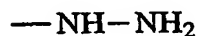
- 2 a) providing a first oligopeptide, the first  
3 oligopeptide having a reactive moiety,  
4 b) (i) providing a precursor oligopeptide  
5 molecule, the precursor oligopeptide molecule  
6 comprising a second oligopeptide fused N-terminally  
7 to an intein domain  
8 (ii) allowing thiol reagent dependent cleavage of  
9 the precursor molecule to generate a second  
10 oligopeptide molecule, said second oligopeptide  
11 molecule having a thioester moiety at its C-terminus  
12 c) allowing the reactive moiety of the first  
13 oligopeptide to react with the second oligopeptide  
14 molecule to form an oligopeptide product, in which  
15 the first and second oligopeptides are linked via a  
16 linking moiety having Formula I, II or III.

17

18 The reactive moiety of the first oligopeptide may be  
19 any suitable reactive moiety. In preferred  
20 embodiments of the invention, the reactive moiety is  
21 a hydrazine moiety, an amino-oxy moiety or a  
22 hydrazide moiety having general formula IV, V or VI  
23 respectively.

24

25 Formula IV

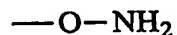


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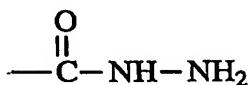
29 Formula V



30

31

1  
2 Formula VI  
3



4  
5 For example, in a particular preferred embodiment,  
6 the reactive moiety has Formula IV and, in the  
7 oligopeptide product produced by the method of the  
8 invention, the first and second oligopeptides are  
9 linked via a linking moiety having Formula I.

10  
11 In a further preferred embodiment, the reactive  
12 moiety has Formula V and, in the oligopeptide  
13 product produced by the method of the invention, the  
14 first and second oligopeptides are linked via a  
15 linking moiety having Formula II.

16  
17 In another preferred embodiment, the reactive moiety  
18 has Formula VI and, in the oligopeptide product  
19 produced by the method of the invention, the first  
20 and second oligopeptides are linked via a linking  
21 moiety having Formula III.

22  
23 As described above, the first oligopeptide comprises  
24 a reactive moiety, which, in preferred embodiments,  
25 may be a hydrazine moiety (e.g. Formula IV), an  
26 amino-oxy moiety (e.g. Formula V) or an hydrazide  
27 moiety (e.g. Formula VI).

28  
29 Hydrazine, hydrazide or aminooxy containing  
30 derivatives of synthetic oligopeptides may be  
31 readily produced using known methods, for example,  
32 solid phase synthesis techniques.

1  
2 Further, the present inventors have also found that  
3 proteins fused N-terminal to an intein domain can be  
4 cleaved from the intein by hydrazine treatment in a  
5 selective manner to liberate the desired protein as  
6 its corresponding hydrazide derivative (for example,  
7 see Figure 5).

8  
9 Accordingly, in further preferred embodiments of the  
10 invention, the first oligopeptide is generated by  
11 reaction of hydrazine with an oligopeptide molecule  
12 comprising the first oligopeptide fused N-terminal  
13 to an intein domain.

14  
15 Indeed the discovery that such protein hydrazides  
16 may be produced by means of such a reaction forms an  
17 independent aspect of the present invention.

18  
19 Accordingly, a third aspect of the invention  
20 provides a method of generating a protein hydrazide,  
21 said method comprising the steps:

22 (a) providing an protein molecule comprising an  
23 oligopeptide fused N-terminal to an intein domain,  
24 (b) reacting said protein molecule with hydrazine,  
25 such that the intein domain is cleaved from the  
26 oligopeptide to generate a protein hydrazide.

27  
28 Moreover, as well as using such a reaction to  
29 generate a first oligopeptide having a hydrazide  
30 moiety at its C-terminal, the first oligopeptide  
31 thus being available for reaction with the second  
32 oligopeptide having the activated ester moiety, the

1 present invention further extends to a "one-step"  
2 process for ligating two peptides to generate an  
3 oligopeptide product.

4

5 This may be achieved by reacting a suitable protein  
6 linked N-terminal to an intein directly with a  
7 polypeptide having a hydrazine, hydrazide or amino-  
8 oxy moiety.

9

10 Accordingly, in a fourth aspect, the invention  
11 provides a method of producing an oligopeptide  
12 product, the method comprising the steps:

- 13 a) providing a first oligopeptide, the first  
14 oligopeptide having a reactive moiety, wherein the  
15 reactive moiety is a hydrazine moiety, a hydrazide  
16 moiety or an amino-oxy moiety;  
17 (i) providing a precursor oligopeptide molecule, the  
18 precursor oligopeptide molecule comprising a second  
19 oligopeptide fused N-terminally to an intein domain;  
20 (c) allowing the reactive moiety of the first  
21 oligopeptide to react with the precursor  
22 oligopeptide molecule to form an oligopeptide  
23 product, in which the first and second oligopeptides  
24 are linked via a linking moiety having Formula I,  
25 Formula II or Formula III.

26

27 The ligation technology of the present invention can  
28 thus utilise both synthetic and recombinant proteins  
29 and peptides. It thus enables the ligation of two or  
30 more synthetic, two or more recombinant or a mixture  
31 of one or more synthetic with one or more  
32 recombinant peptides.

1  
2 Moreover, as well as providing a novel method of  
3 ligating peptides, the present invention may be used  
4 for the labelling of synthetic or recombinant  
5 peptides.

6  
7 Accordingly, in a fifth aspect of the present  
8 invention, there is provided a method of labelling  
9 an oligopeptide, the method comprising the steps:  
10 a) providing a label molecule, the label molecule  
11 having a reactive moiety,  
12 b) providing the oligopeptide, the oligopeptide  
13 having an activated ester moiety  
14 c) allowing the reactive moiety of the label  
15 molecule to react with the activated ester moiety of  
16 the oligopeptide to form the labelled oligopeptide,  
17 in which the label molecule and the oligopeptide are  
18 linked via a linking moiety having Formula I,  
19 Formula II or Formula III as defined above,

20  
21 In preferred embodiments, in step (c), where said  
22 label molecule and the oligopeptide are linked via a  
23 linking moiety having Formula II and where said  
24 activated ester moiety of step (b) is not a  
25 thioester, said activated ester is a terminal  
26 activated ester moiety.

27  
28 Alternatively, a label molecule having a terminal  
29 activated ester moiety may be used to label an  
30 oligopeptide having a reactive moiety. Thus, in a  
31 sixth aspect of the invention, there is provided a  
32 method of labelling an oligopeptide, the method

1 comprising the steps:

- 2 a) providing a label molecule, the label molecule  
3 having an activated ester moiety of which the label  
4 is the acyl substituent,  
5 b) providing the oligopeptide, the oligopeptide  
6 having a reactive moiety  
7 c) allowing the activated ester moiety of the label  
8 molecule to react with the reactive moiety of the  
9 oligopeptide to form the labelled oligopeptide, in  
10 which the label molecule and the oligopeptide are  
11 linked via a linking moiety having Formula I,  
12 Formula II or Formula III

13 wherein, in step (c), where said label molecule  
14 and the oligopeptide are linked via a linking moiety  
15 having Formula II and where said activated ester  
16 moiety of step (b) is not a thioester, said  
17 activated ester is a terminal activated ester  
18 moiety.

19

20 As with the ligation technology, an oligopeptide  
21 present as a precursor molecule linked to an intein  
22 molecule may be labelled directly. Thus, a seventh  
23 aspect of the present invention provides a method of  
24 labelling an oligopeptide, the method comprising the  
25 steps:

- 26 a) providing a label molecule, the label molecule  
27 having a reactive moiety,  
28 b) providing a precursor oligopeptide molecule,  
29 the precursor oligopeptide molecule comprising an  
30 oligopeptide fused N-terminally to an intein domain,  
31 c) allowing the reactive moiety of the label  
32 molecule to react with the precursor oligopeptide

1 molecule to form a labelled oligopeptide product, in  
2 which the label molecule and the oligopeptide are  
3 linked via a linking moiety having Formula I,  
4 Formula II or Formula III as defined above.

5  
6 The methods of the invention are particularly useful  
7 in the ligation of peptides, in particular the  
8 ligation of peptides, which, using conventional  
9 ligation techniques, would require various  
10 protecting groups. The inventors have shown that  
11 the methods of the invention may be performed under  
12 pH conditions in which only the reactive moieties  
13 will react.

14  
15 In preferred embodiments of the first to seventh  
16 aspects of the invention, the method is performed at  
17 a pH in the range pH 4.0 to pH 8.5, preferably pH  
18 4.0 to 7.5, more preferably in the range pH 4.5 to  
19 pH 7.0, most preferably in the range pH 5.5 to pH  
20 6.5.

21  
22 For example, the inventors have demonstrated that  
23 synthetic peptide C-terminal thioesters specifically  
24 react with hydrazine under aqueous conditions at pH  
25 6.0 to form the corresponding peptide hydrazide.  
26 This allows ligation methods as described herein to  
27 be performed at pH 6.0, without the need for a  
28 potentially harmful thiol cofactor (useful if either  
29 fragment or final construct is thiol sensitive) and  
30 does not lead to the introduction of potentially  
31 reactive side-chain groups (such as a thiol) into  
32 the protein. Similarly, the inventors have

1 demonstrated that synthetic peptide C-terminal  
2 thioesters specifically react with hydroxylamine  
3 under aqueous conditions at pH 6.0 and pH 6.8 to  
4 form the corresponding peptide hydroxamic acid.

5  
6 In an analogous fashion, peptides that contain  
7 hydrazine, hydrazide or aminooxy groups can be  
8 reacted with thioester derivatives of a label or a  
9 peptide to afford site-specific labelling and  
10 chemoselective ligation respectively (see, for  
11 example, figures 4 and 5).

12  
13 Furthermore, having demonstrated that recombinant  
14 protein hydrazides can be generated by cleavage of  
15 protein-intein fusions with hydrazine, the inventors  
16 have shown that such protein hydrazides may be  
17 ligated by reaction of the hydrazide moiety with  
18 reactive groups other than activated ester moieties,  
19 for example an aldehyde functionality, a ketone  
20 functionality or an isocyanate functionality. This  
21 aspect of the invention provides a further novel  
22 method of ligating a recombinant peptide to a second  
23 peptide or indeed a label.

24  
25 Thus, an eighth aspect of the invention provides a  
26 method of producing an oligopeptide product, the  
27 method comprising the steps:

- 28 a) providing a first oligopeptide, the the first
- 29 oligopeptide having an aldehyde or ketone moiety,
- 30 b) providing a precursor oligopeptide molecule,
- 31 the precursor oligopeptide molecule comprising a
- 32 second oligopeptide fused N-terminally to an intein



1 domain,  
2 c) reacting said precursor oligopeptide molecule  
3 with hydrazine to generate an oligopeptide molecule  
4 comprising an intermediate oligopeptide , said  
5 intermediate oligopeptide having a C-terminal  
6 hydrazide moiety,  
7 d) allowing the aldehyde or ketone moiety of the  
8 first oligopeptide to react with the hydrazide  
9 moiety of the intermediate oligopeptide molecule to  
10 form an oligopeptide product, in which first  
11 oligopeptide and the second oligopeptide are linked  
12 via a hydrazone linking moiety.  
13  
14 An example of this aspect is shown in Figure 6.  
15  
16 A ninth aspect of the invention provides a method of  
17 labelling an oligopeptide, the method comprising the  
18 steps:  
19 a) providing a label molecule, the label molecule  
20 having a aldehyde or ketone moiety,  
21 b) providing a precursor oligopeptide molecule,  
22 the precursor oligopeptide molecule comprising a  
23 first oligopeptide fused N-terminally to an intein  
24 domain,  
25 c) reacting said precursor oligopeptide molecule  
26 with hydrazine to generate an oligopeptide molecule  
27 comprising an intermediate oligopeptide , said  
28 intermediate oligopeptide having a terminal  
29 hydrazide moiety,  
30 d) allowing the aldehyde or ketone moiety of the  
31 label molecule to react with the hydrazide moiety of  
32 the intermediate oligopeptide molecule to form a

1 labelled oligopeptide product, in which the label  
2 molecule and oligopeptide are linked via a hydrazone  
3 linking moiety.

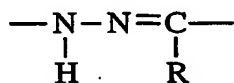
4

5 In preferred embodiments of the eighth and ninth  
6 aspects of the invention, the hydrazone moiety has  
7 Formula VII:

8

9

10



11

12 where R is H or any substituted or unsubstituted,  
13 preferably unsubstituted, alkyl group.

14

15 In preferred aspects of the eighth and ninth aspects  
16 of the invention, the method is performed at a pH in  
17 the range pH 1.0 to pH 7.0, preferably pH 1.0 to pH  
18 6.0, more preferably in the range pH 2.0 to pH 5.5,  
19 most preferably in the range pH 2.0 to pH 4.5.

20

21 In a tenth aspect of the present invention, there is  
22 provided an oligopeptide product produced using a  
23 method of the invention.

24

25 In an eleventh aspect, there is provided a labelled  
26 oligopeptide comprising an oligopeptide labelled  
27 according to a method of the invention.

28

29 Preferred features of each aspect of the invention  
30 are as for each of the other aspects mutatis  
31 mutandis.

32

1 The invention will now be described further in the  
2 following non-limiting examples with reference made  
3 to the accompanying drawings in which:

4

5 Figure 1 illustrates schematically the general  
6 principle of chemical ligation.

7

8 Figure 2 illustrates schematically the mechanism of  
9 protein splicing.

10

11 Figure 3 illustrates the generation of recombinant  
12 C-terminal thioester proteins.

13

14 Figure 4 illustrates ligation of protein and peptide  
15 thioesters with hydrazine and aminooxy containing  
16 entities, such as labels, peptides and proteins.

17

18 Figure 5 illustrates the generation of synthetic and  
19 recombinant peptide hydrazides for ligation with  
20 thioester containing molecules. Note the peptide or  
21 label is the acyl substituent of the thioester.

22

23 Figure 6 illustrates the generation of recombinant  
24 peptide hydrazides for ligation with aldehyde and  
25 ketone containing molecules.

26

27 Figure 7 illustrates SDS-PAGE analysis of Grb2-SH2 -  
28 GyrA - CBD (immobilised on chitin beads) treated  
29 with DTT and MESNA. Molecular weight markers (lane  
30 1); purified Grb2-SH2 - GyrA - CBD immobilised on  
31 chitin beads (lane 4). Grb2-SH2 - GyrA - CBD treated  
32 with 100 mM DTT (lanes 5 and 7) or 120 mM MESNA

1 (lanes 8 and 10). Both the whole reaction slurries  
2 (lanes 5 and 8) and the reaction supernatants (lanes  
3 7 and 10) were analysed.

4

5 Figure 8 illustrates SDS-PAGE analysis of Grb2-SH2 -  
6 GyrA - CBD (immobilised on chitin beads) treated  
7 with hydrazine. Molecular weight markers (lane 1);  
8 Purified Grb2-SH2 - GyrA - CBD immobilised on chitin  
9 beads after 20h treatment with phosphate buffer only  
10 (lane 2). Grb2-SH2 - GyrA - CBD treated with 200 mM  
11 hydrazine in phosphate buffer for 20 h. The whole  
12 reaction slurries were analysed.

13

14 Figure 9 illustrates an ESMS spectrum of the C-  
15 terminal hydrazide derivative of Grb2-SH2.

16

17 Figure 10 shows SDS-PAGE analysis of the reaction  
18 between synthetic ketone containing peptide CH<sub>3</sub>COCO-  
19 myc with Grb2-SH2 - C-terminal hydrazide and  
20 Cytochrome C. Molecular weight markers (lane 1);  
21 Grb2-SH2 - C-terminal DTT thioester (lane 2).  
22 Reaction between Grb2-SH2 - C-terminal hydrazide and  
23 CH<sub>3</sub>COCO-myc at time points t=0 h (lane 3), t=24 h  
24 (lane 4), t= 48h (lane 5) and t= 72 h (lanes 6).  
25 Reaction between Cytochrome C and CH<sub>3</sub>COCO-myc at  
26 time points t=0 h (lane 7), t=24 h (lane 8), t= 48h  
27 (lane 9) and t= 72 h (lanes 10)

28

29

30 **Examples**

31

1     Example 1 -Protein ligation / site specific protein  
2     labelling using the reaction of peptide / protein  
3     thioesters with compounds containing hydrazine /  
4     hydrazide or aminoxy functionalities.

5  
6     Reaction of a peptide C-terminal thioester with  
7     100mM hydrazine at pH 6.0  
8     200 mM sodium phosphate buffer pH 6.0 containing  
9     100mM hydrazine monohydrate (200  $\mu$ L) was added to a  
10    model synthetic peptide  $\alpha$ -thioester termed AS626p1A  
11    (200  $\mu$ g) to yield a final peptide concentration of  
12    317  $\mu$ M. AS626p1A has sequence ARTKQ TARK(Me)<sub>3</sub>  
13    STGGKAPRKQ LATKAARK-COS-(CH<sub>2</sub>)<sub>2</sub>-COOC<sub>2</sub>H<sub>5</sub> (SEQ ID NO: 1)  
14    wherein a single Alanine residue (which may be any  
15    one of the Alanine residues of SEQ ID NO: 1) is  
16    substituted by an Arginine residue. The reaction was  
17    incubated at room temperature and monitored with  
18    time by analytical reversed phase HPLC. Vydac C18  
19    column (5  $\mu$ M, 0.46 x ). Linear gradients of  
20    acetonitrile water / 0.1% TFA were used to elute the  
21    peptides at a flow rate of 1 mL min<sup>-1</sup>. Individual  
22    peptides eluting from the column were characterised  
23    by electrospray mass spectrometry.

24  
25    Reaction of a peptide C-terminal thioester with  
26    100mM hydroxylamine at pH 6.0  
27    200 mM sodium phosphate buffer pH 6.0 containing  
28    100mM hydroxylamine hydrogen chloride (200  $\mu$ L) was  
29    added to AS626p1A (200  $\mu$ g) to yield a final peptide  
30    concentration of 317  $\mu$ M. The reaction was incubated  
31    at room temperature and monitored with time by

1 analytical reversed phase HPLC. Vydac C18 column (5  
2  $\mu\text{M}$ , 0.46 x ). Linear gradients of acetonitrile water  
3 / 0.1% TFA were used to elute the peptides at a flow  
4 rate of 1 mL min<sup>-1</sup>. Individual peptides eluting from  
5 the column were characterised by electrospray mass  
6 spectrometry.

7

8 *Reaction of a peptide C-terminal thioester with 100*  
9 *mM hydroxylamine at pH 6.8*

10 200 mM sodium phosphate buffer pH 6.8 containing  
11 100mM hydroxylamine hydrogen chloride (200  $\mu\text{L}$ ) was  
12 added to AS626p1A (200  $\mu\text{g}$ ) to yield a final peptide  
13 concentration of 317  $\mu\text{M}$ . The reaction was incubated  
14 at room temperature and monitored with time by  
15 analytical reversed phase HPLC. Vydac C18 column (5  
16  $\mu\text{M}$ , 0.46 x ). Linear gradients of acetonitrile water  
17 / 0.1% TFA were used to elute the peptides at a flow  
18 rate of 1 mL min<sup>-1</sup>. Individual peptides eluting from  
19 the column were characterised by electrospray mass  
20 spectrometry.

21

22 *Reaction of a peptide C-terminal thioester with 10mM*  
23 *hydroxylamine at pH 6.8*

24 See above procedure.

25

26 *Reaction of a peptide C-terminal thioester with 10mM*  
27 *hydroxylamine at pH 7.5*

28 See above procedure.

29

30 *Reaction of a peptide C-terminal thioester with 2mM*  
31 *hydroxylamine at pH 7.5*

1 See above procedure.

2

### 3 Results

4 These examples demonstrate the novel strategy for  
5 protein ligation / site specific protein labelling  
6 of both synthetic and recombinant protein sequences  
7 of the invention using the reaction of peptide /  
8 protein C-terminal thioesters with compounds  
9 containing hydrazine / hydrazide or aminoxy  
10 functionalities.

11

12 As described above, a purified synthetic 27 amino  
13 acid  $\alpha$ -thioester peptide (the ethyl 3-  
14 mercaptopropionate thioester derivative) was treated  
15 with hydrazine and hydroxylamine under various  
16 conditions (Table 1).

17

18 Treatment with 100 mM hydrazine at pH 6.0 formed a  
19 peptide species that eluted earlier than the  
20 starting thioester peptide as analysed by HPLC. This  
21 material was identified as the expected peptide  
22 hydrazide by ESMS: observed mass = 3054 Da, expected  
23 (av. isotope comp) 3053 Da. The reaction of the  
24 peptide C-terminal thioester with hydrazine to form  
25 the peptide hydrazide was monitored with time by  
26 reverse phase HPLC. Only the desired material was  
27 formed with no side product formation even after 3  
28 days. The stability of the peptide hydrazide, under  
29 the reaction conditions, indicates that the reaction  
30 occurs at the C-terminal thioester moiety and is  
31 chemoselective in nature. It also highlights the  
32 applicability of this reaction for protein ligation

1 and labelling. (2 h 70% conversion , 4h 95%  
2 conversion)

3

4 To ascertain whether aminooxy containing compounds  
5 chemoselectively react with peptide / protein C-  
6 terminal thioesters, to afford protein ligation and  
7 site-specific labelling, a synthetic C-terminal  
8 thioester peptide was treated with hydroxylamine  
9 under various conditions (Table 1).

10

11 A purified synthetic 27 amino acid C-terminal  
12 thioester peptide (ethyl 3-mercaptopropionate  
13 thioester, observed mass 3155 Da) was incubated at  
14 room temperature with different hydroxylamine  
15 concentrations in aqueous buffers of varying pH. In  
16 all cases the peptide C-terminal thioester reacted  
17 to form a single product that eluted earlier than  
18 the starting thioester peptide as analysed by  
19 reverse phase HPLC. This material corresponds to the  
20 expected hydroxamic acid peptide as determined by  
21 ESMS: observed mass = 3052 Da, expected (av. isotope  
22 comp) 3054 Da. The kinetics of the reaction were  
23 monitored using reverse phase HPLC. The peptide C-  
24 terminal thioester is converted to the corresponding  
25 peptide hydroxamic acid in a clean fashion with no  
26 side-product formation. As expected increasing the  
27 pH of the reaction buffer accelerates the rate of  
28 reaction. With 100mM  $\text{NH}_2\text{OH}$  on moving from pH 6.0 to  
29 pH 6.8 the percentage product formation after 1h  
30 increases from 25% to 91%. The rate of reaction with  
31 100 mM  $\text{NH}_2\text{OH}$  pH 6.0 is comparable with 10 mM  $\text{NH}_2\text{OH}$  at  
32 pH 6.8.



1  
2 The rate of reaction of the peptide C-terminal  
3 thioester with hydroxalymine, to form the  
4 corresponding hydroxamic acid, increases with  
5 increasing pH and decreases with decreasing  $\text{NH}_2\text{OH}$   
6 concentrations. To identify conditions of pH and  
7 reactant concentration suitable for peptide /  
8 protein labelling and ligation, the labelling was  
9 performed under increasing pH and decreasing  $\text{NH}_2\text{OH}$   
10 concentrations.

11  
12 The reaction with 10 mM was 83% complete after 4h at  
13 pH 6.8, while at pH 7.5 it was 83% complete after  
14 2h. On further decreasing the  $\text{NH}_2\text{OH}$  concentration to  
15 2 mM the reaction rate at pH 7.5 decreased markedly,  
16 70% of the starting peptide  $\alpha$ -thioester being  
17 converted to the corresponding hydroxamic acid after  
18 8hrs. It was noted that a small amount of a side-  
19 product corresponding in mass to the peptide acid  
20 was formed during the reaction. Presumably this is  
21 formed by a competing hydrolysis side reaction at pH  
22 7.5, which was not observed with 10 mM  $\text{NH}_2\text{OH}$  at pH  
23 7.5 due to the faster reaction at this higher  
24 reactant concentration.

Reactant	Concentration	pH	Percentage product formation with time				
			1hr	2hr	4hr	8hr	72hr
NH <sub>2</sub> NH <sub>2</sub>	100 mM	6.0	-	70	100		
NH <sub>2</sub> OH	100 mM	6.0	25	48.1	76.3	-	100
NH <sub>2</sub> OH	100 mM	6.8	91	100			
NH <sub>2</sub> OH	10 mM	6.8	26	-	83	100	
NH <sub>2</sub> OH	10 mM	7.5	-	82.7	100	100	
NH <sub>2</sub> OH	2 mM	7.5	11.2	17	38	70	80*

**Table 1**

\*All starting material has reacted with 80% conversion to the desired product and ~20% to the hydrolysis side-product.

**Example 2- Generation of recombinant C-terminal hydrazide proteins through the selective cleavage of protein - intein fusions with hydrazine, and their subsequent use in ligation / labelling reactions.**

To investigate (i) the ability to generate recombinant C-terminal hydrazide proteins through the selective cleavage of protein - intein fusions with hydrazine, and (ii) their subsequent use in ligation / labelling reactions, the SH2 domain of the adapter protein Grb2 was chosen as a model system.

Sequence of human Grb2 SH2 domain

HPW FFGKIPRAKA EEMLSKQRHD GAFLIRESES APGDFSLSVK  
FGNDVQHFKV LRDGAGKYFL WVKFNSLNE LVDYHRSTSV  
SRNQQIFLRD IEQVPQQPT

1     *Expression of Grb2-SH2 domain - GyrA intein fusion.*

2             The DNA sequence encoding the SH2 domain of  
3     human Grb2 appended at its C-terminus with an extra  
4     glycine residue was cloned into the pTXB1 expression  
5     plasmid (NEB). This vector pTXB1<sub>Grb2-SH2 (Gly)</sub> encodes  
6     for a fusion protein whereby the SH2 domain of Grb2  
7     is linked via a glycine residue to the N-terminus of  
8     the GyrA intein, which is in turn fused to the N-  
9     terminus of a chitin binding domain region (CBD).  
10    *E. coli* cells were transformed with this plasmid and  
11    grown in LB medium to mid log phase and protein  
12    expression induced for 4h at 37°C with 0.5 mM IPTG.  
13    After centrifugation the cells were re-suspended in  
14    lysis buffer (0.1 mM EDTA, 250 mM NaCl, 5% glycerol,  
15    1 mM PMSF, 25 mM HEPES, pH 7.4) and lysed by  
16    sonication. The soluble fraction was loaded onto a  
17    chitin column pre- equilibrated in lysis buffer. The  
18    column was then washed with wash buffer (1 mM EDTA,  
19    250 mM NaCl, 0.1% Triton-X 100, , 25 mM HEPES, pH  
20    7.0) to yield purified Grb2-SH2 - GyrA-CBD  
21    immobilised on chitin beads (Figure 7).

22  
23     *Generation of Grb2-SH2 C-terminal thioesters by*  
24     *thiol induced cleavage of the Grb2-SH2 - GyrA intein*  
25     *fusion.*

26     To ascertain that the intein domain within the  
27     protein was functional the fusion protein was  
28     exposed to thiols to assess the extent of cleavage  
29     via transthioesterification. Chitin beads containing  
30     immobilised Grb2-SH2 - GyrA-CBD were equilibrated  
31     into 200 mM NaCl, 200 mM phosphate buffer pH 7.4.  
32     Dithiothreitol (DTT) or 2-mercaptoethanesulfonic

1 acid (MESNA) were then added to the beads in 200 mM  
2 NaCl, 200 mM phosphate buffer pH 7.4 to give a 50%  
3 slurry with a final thiol concentration of 100 mM or  
4 120 mM respectively. The mixtures were then rocked  
5 at room temperature and aliquots analysed by SDS-  
6 PAGE. After 48 hours the supernatants from the  
7 reactions were isolated and subsequently analysed by  
8 HPLC and ESMS.

9 Treatment of Grb2-SH2 - GyrA intein - CBD  
10 fusion with both DTT and MESNA resulted in cleavage  
11 of the fusion protein into two protein species  
12 (Figure 7). The molecular size of the two fragments  
13 corresponds to that of the Grb2 - SH2 and the GyrA -  
14 intein fusion, indicative that cleavage has taken  
15 place at the SH2 - intein junction. Cleavage of the  
16 precursor fusion protein liberated the SH2 domain  
17 into the supernatant while the GyrA intein-CBD  
18 portion remained immobilized on the chitin beads.  
19 After cleavage with both DTT or MESNA, ESMS analysis  
20 of the supernatants confirmed that the Grb2-SH2 was  
21 generated as either the expected DTT or MESNA C-  
22 terminal thioester derivatives respectively.

23 Expected mass of Grb2-SH2 DTT - C-terminal  
24 thioester = 12173.9 Da; observed mass 12173.5 Da.  
25 Expected mass of Grb2-SH2 MESNA - C-terminal  
26 thioester = 12162.0 Da; observed mass 12163.0 Da.

27

28 *Generation of Grb2-SH2 C-terminal hydrazide by*  
29 *hydrazine induced cleavage of the Grb2-SH2 - GyrA*  
30 *intein fusion.*

31

1           The thioester linkage between Grb2-SH2 and the  
2   GyrA intein in the precursor fusion protein is  
3   expected to be cleaved with hydrazine, the  
4   chemoselective reaction of hydrazine, at the  
5   thioester moiety, liberating Grb2-SH2 domain into  
6   the supernatant as its corresponding C-terminal  
7   hydrazide derivative. Chitin beads containing  
8   immobilised Grb2-SH2 - GyrA-CBD were therefore  
9   equilibrated into 200 mM NaCl, 200 mM phosphate  
10   buffer pH 7.4 and hydrazine monohydrate added in the  
11   same buffer to give a 50% slurry with a final  
12   hydrazine concentration of 200 mM. The mixture was  
13   then rocked at room temperature and analysed by SDS-  
14   PAGE (Figure 8). After 20 hours the supernatant was  
15   removed and analysed by HPLC and ESMS.

16           Treatment of Grb2-SH2 - GyrA intein - CBD  
17   fusion with hydrazine resulted in cleavage of the  
18   fusion protein into two species. The molecular size  
19   of the two fragments as analysed by SDS-PAGE  
20   corresponded to Grb2 - SH2 and the GyrA - intein  
21   fusion, indicative that cleavage has taken place at  
22   the unique thioester linkage between the SH2 -  
23   intein domains. Cleavage of the precursor fusion  
24   protein liberates the SH2 domain into the  
25   supernatant while the GyrA intein-CBD portion  
26   remained immobilized on the chitin beads. HPLC and  
27   ESMS analysis of the cleavage supernatant confirmed  
28   that a single protein species was generated that  
29   corresponds to the C-terminal hydrazide derivative  
30   of Grb2-SH2. Expected mass of Grb2-SH2 C-terminal  
31   hydrazide = 12051.7 Da; observed mass 12053.0 Da.  
32   (Figure 9)

1  
2 After 20 h of reaction Grb2-SH2 C-terminal hydrazide  
3 was isolated from the supernatant using RPHPLC and  
4 lyophilised.

5  
6 *Ligation of aldehyde and ketone containing peptides*  
7 *and labels to recombinant C-terminal hydrazide*  
8 *containing proteins.*

9  
10 It was anticipated that recombinant protein C-  
11 terminal hydrazides, generated by hydrazine  
12 treatment of the corresponding intein fusion  
13 precursor, can be site-specifically modified by  
14 chemoselective ligation with aldehyde and ketone  
15 containing peptides and labels. To demonstrate such  
16 an approach the ability of a synthetic ketone  
17 containing peptide to ligate with the Grb2-SH2 C-  
18 terminal hydrazide generated above was investigated.  
19 A synthetic peptide corresponding to the c-myc  
20 epitope sequence was synthesised GEQKLISEEDL-NH<sub>2</sub>  
21 whereby pyruvic acid was coupled to the amino  
22 terminus of the peptide as the last step of the  
23 assembly. This peptide (designated CH<sub>3</sub>COCO-myc) was  
24 purified to > 95% purity by RPHPLC and lyophilised  
25 (ESMS expected monoisotopic mass 1328.6 Da; observed  
26 mass 1328.6 Da).

27 A sample of CH<sub>3</sub>COCO-myc peptide was dissolved  
28 in 100 mM sodium acetate buffer pH 4.5 to give a 4  
29 mM peptide concentration. This peptide solution (100  
30 µL) was then added to an aliquot of lyophilised  
31 Grb2-SH2 C-terminal hydrazide protein (~ 250 µg) and  
32 the reaction monitored by SDS-PAGE (Figure 10) As a

1 control CH<sub>3</sub>COCO-myc was also incubated with  
2 Cytochrome C, a protein of similar same size to  
3 Grb2-SH2 but absent of a hydrazide functionality.

4 SDS-PAGE analysis showed that CH<sub>3</sub>COCO-myc  
5 peptide has indeed ligated with Grb2-SH2 C-terminal  
6 hydrazide as indicated by the conversion of Grb2-  
7 SH2 C-terminal hydrazide into a protein species of  
8 a higher molecular weight (approximately 1000-2000  
9 Da higher). The reaction was virtually complete  
10 after 24 h and the reaction product appeared to be  
11 stable. On the other hand there was no observable  
12 change to Cytochrome C with time i.e no ligation,  
13 establishing that the ligation reaction was  
14 occurring at the C-terminal hydrazide functionality  
15 of Grb2-SH2.

16 After 96 h of reaction the product from the  
17 Grb2-SH2 ligation reaction was isolated by HPLC and  
18 characterised by ESMS. Chemosselective ligation of  
19 CH<sub>3</sub>COCO-myc to Grb2-SH2 C-terminal hydrazide via  
20 hydrazone bond formation would give a product of  
21 expected mass 13363.7 Da. The observed product mass  
22 was 13364.1 Da indicating that the desired ligation  
23 product had been formed.

24  
25 In summary, the present invention provides novel  
26 methods of protein ligation that enable both  
27 synthetic and recombinantly derived protein  
28 fragments to be efficiently joined together in a  
29 regioselective manner. This thus enables large  
30 proteins to be constructed from combinations of  
31 synthetic and recombinant fragments and allows  
32 proteins of any size to be site-specifically

1 modified in an unprecedented manner. This is of  
2 major importance for biological and biomedical  
3 science and drug discovery when one considers that  
4 the ~ 30,000 human genes yield hundreds of thousands  
5 of different protein species through post-  
6 translational modification. Such post-  
7 translationally modified proteins cannot be accessed  
8 through current recombinant technologies.

9  
10 The application of such protein ligation techniques  
11 may be used for protein based tools, protein  
12 therapeutics and in *de novo* design and may open up  
13 many new avenues in biological and biomedical  
14 sciences that have hitherto not been possible.

15  
16 All documents referred to in this specification are  
17 herein incorporated by reference. Various  
18 modifications and variations to the described  
19 embodiments of the inventions will be apparent to  
20 those skilled in the art without departing from the  
21 scope and spirit of the invention. Although the  
22 invention has been described in connection with  
23 specific preferred embodiments, it should be  
24 understood that the invention as claimed should not  
25 be unduly limited to such specific embodiments.  
26 Indeed, various modifications of the described modes  
27 of carrying out the invention which are obvious to  
28 those skilled in the art are intended to be covered  
29 by the present invention.

30

31



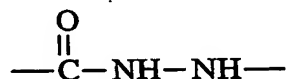
1     **Claims**

2

- 3     1.    A method of producing an oligopeptide product,  
 4     the method comprising the steps:  
 5     a)    providing a first oligopeptide, the first  
 6     oligopeptide having a reactive moiety,  
 7     b)    providing a second oligopeptide, the second  
 8     oligopeptide having a activated ester moiety  
 9     c)    allowing the reactive moiety of the first  
 10    oligopeptide to react with the activated ester  
 11    moiety of the second oligopeptide to form an  
 12    oligopeptide product, in which the first and second  
 13    oligopeptides are linked via a linking moiety having  
 14    Formula I, Formula II or Formula III.

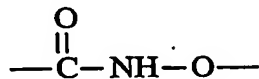
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16           Formula I



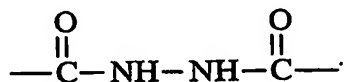
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18           Formula II



19

20           Formula III



21

22

23

- 24     2. The method according to claim 1 wherein the  
 25     terminal activated ester moiety is a thioester  
 26     wherein the peptide is the acyl substituent of

1 the thioester.

2

3 3. The method according to claim 2, wherein said  
4 second polypeptide is generated by thiol reagent  
5 dependent cleavage of a precursor molecule, said  
6 precursor molecule comprising a second oligopeptide  
7 fused N-terminally to an intein domain.

8

9 4. A method of producing an oligopeptide product,  
10 the method comprising the steps:

11 a) providing a first oligopeptide, the first  
12 oligopeptide having a reactive moiety,  
13 (i) providing a precursor oligopeptide molecule, the  
14 precursor oligopeptide molecule comprising a second  
15 oligopeptide fused N-terminally to an intein domain  
16 (ii) allowing thiol reagent dependent cleavage of  
17 the precursor molecule to generate a second  
18 oligopeptide molecule, said second oligopeptide  
19 molecule having a thioester moiety at its C-  
20 terminus,  
21 c) allowing the reactive moiety of the first  
22 oligopeptide to react with the second oligopeptide  
23 molecule to form an oligopeptide product, in which  
24 the first and second oligopeptides are linked via a  
25 linking moiety having Formula I, II or III.

26

27 5. The method according to any one of the preceding  
28 claims wherein the reactive moiety is a hydrazine  
29 moiety, a hydrazide moiety or an aminooxy moiety.

30

31 6. The method according to claim 5, wherein said  
32 first oligopeptide is produced by reaction of

1 hydrazine with a precursor molecule, said  
2 precursor molecule comprising a precursor  
3 oligopeptide fused N-terminally to an intein  
4 domain via a thioester moiety.  
5

6 7. A method of producing an oligopeptide product,  
7 said method comprising the steps:  
8 a) providing a first oligopeptide, the first  
9 oligopeptide having a reactive moiety, wherein  
10 the reactive moiety is a hydrazine moiety, a  
11 hydrazide moiety or an amino-oxy moiety;  
12 (i) providing a precursor oligopeptide molecule,  
13 the precursor oligopeptide molecule comprising a  
14 second oligopeptide fused N-terminally to an  
15 intein domain;  
16 (c) allowing the reactive moiety of the first  
17 oligopeptide to react with the precursor  
18 oligopeptide molecule to form an oligopeptide  
19 product, in which the first and second  
20 oligopeptides are linked via a linking moiety  
21 having Formula I, Formula II or Formula III.  
22

23 8. The method according to any one of the preceding  
24 claims, wherein the first oligopeptide or the  
25 second oligopeptide is a recombinant oligopeptide  
26 and the other of the the first oligopeptide and  
27 the second oligopeptide is a synthetic  
28 polypeptide.  
29

30 9. The method according to any one of claims 1 to 7,  
31 wherein the first oligopeptide and the second

1        oligopeptide are recombinant oligopeptides.

2

3        10. The method according to any one of claims 1 to  
4        7, wherein the first oligopeptide and the second  
5        oligopeptide are synthetic oligopeptides.

6

7        11. A method of generating a protein hydrazide,  
8        said method comprising the steps:  
9        (a) providing a protein molecule comprising an  
10       oligopeptide fused N-terminal to an intein  
11       domain,  
12       (b) reacting said protein molecule with  
13       hydrazine, such that the intein domain is cleaved  
14       from the oligopeptide to generate a protein  
15       hydrazide.

16

17       12. The method according to any one of the  
18       preceding claims wherein the method is performed  
19       at a pH in the range pH 5.5 to 7.5.

20

21       13. A method of producing an oligopeptide product,  
22       the method comprising the steps:  
23       a) providing a first oligopeptide, the the first  
24       oligopeptide having an aldehyde or ketone moiety,  
25       b) providing a precursor oligopeptide molecule,  
26       the precursor oligopeptide molecule comprising a  
27       second oligopeptide fused N-terminally to an  
28       intein domain,  
29       c) reacting said precursor oligopeptide molecule  
30       with hydrazine to generate an oligopeptide  
31       molecule comprising an intermediate oligopeptide  
32       , said intermediate oligopeptide having a

- 1 terminal hydrazide moiety,  
2 d) allowing the aldehyde or ketone moiety of the  
3 first oligopeptide to react with the hydrazide  
4 moiety of the intermediate oligopeptide molecule  
5 to form an oligopeptide product, in which first  
6 oligopeptide and the second oligopeptide are  
7 linked via a hydrazone linking moiety.  
8
- 9 14. An oligopeptide product produced by the method  
10 of any one of the preceding claims.  
11
- 12 15. A method of labelling an oligopeptide, the  
13 method comprising the steps:  
14 a) providing a label molecule, the label molecule  
15 having a reactive moiety,  
16 b) providing the oligopeptide, the oligopeptide  
17 having a activated ester moiety  
18 c) allowing the reactive moiety of the label  
19 molecule to react with the activated ester moiety  
20 of the oligopeptide to form the labelled  
21 oligopeptide, in which the label molecule and the  
22 oligopeptide are linked via a linking moiety  
23 having Formula I, Formula II or Formula III.  
24
- 25 16. The method according to claim 15, wherein in  
26 step (c), where said label molecule and the  
27 oligopeptide are linked via a linking moiety  
28 having Formula II and where said activated ester  
29 moiety of step (b) is not a thioester, said  
30 activated ester is a terminal activated ester  
31 moiety.  
32

1     17. A method of labelling an oligopeptide, the  
2       method comprising the steps:  
3       a) providing a label molecule, the label molecule  
4       having an activated ester moiety of which the  
5       label is the acyl substituent,  
6       b) providing the oligopeptide, the oligopeptide  
7       having a reactive moiety  
8       c) allowing the activated ester moiety of the  
9       label molecule to react with the reactive moiety  
10      of the oligopeptide to form the labelled  
11      oligopeptide, in which the label molecule and the  
12      oligopeptide are linked via a linking moiety  
13      having Formula I, Formula II or Formula III,  
14      wherein, in step (c), where said label molecule  
15      and the oligopeptide are linked via a linking  
16      moiety having Formula II and where said activated  
17      ester moiety of step (b) is not a thioester, said  
18      activated ester is a terminal activated ester  
19      moiety.

20  
21     18. A method of labelling an oligopeptide, the  
22       method comprising the steps:  
23       a) providing a label molecule, the label molecule  
24       having a reactive moiety,  
25       b) providing a precursor oligopeptide molecule,  
26       the precursor oligopeptide molecule comprising an  
27       oligopeptide fused N-terminally to an intein  
28       domain,  
29       c) allowing the reactive moiety of the label  
30       molecule to react with the precursor oligopeptide  
31       molecule to form a labelled oligopeptide product,  
32       in which the label molecule and the oligopeptide

1 are linked via a linking moiety having Formula I,  
2 Formula II or Formula III as defined above.

3

4 19. The method according to any one of claims 15 to  
5 18 wherein the method is performed at a pH in the  
6 range pH 5.5 to pH 7.5.

7

8 20. A method of labelling an oligopeptide, the  
9 method comprising the steps:  
10 a) providing a label molecule, the label molecule  
11 having a aldehyde or ketone moiety,  
12 b) providing a precursor oligopeptide molecule,  
13 the precursor oligopeptide molecule comprising a  
14 first oligopeptide fused N-terminally to an  
15 intein domain,  
16 c) reacting said precursor oligopeptide molecule  
17 with hydrazine to generate an oligopeptide  
18 molecule comprising an intermediate oligopeptide,  
19 said intermediate oligopeptide having a terminal  
20 hydrazide moiety,  
21 d) allowing the aldehyde or ketone moiety of the  
22 label molecule to react with the hydrazide moiety  
23 of the intermediate oligopeptide molecule to form  
24 a labelled oligopeptide product, in which the  
25 label molecule and oligopeptide are linked via a  
26 hydrazone linking moiety.

27

28 21. A labelled oligopeptide produced by the method  
29 of any one of claims 15 to 20.

30

31

## Mutually reactive groups

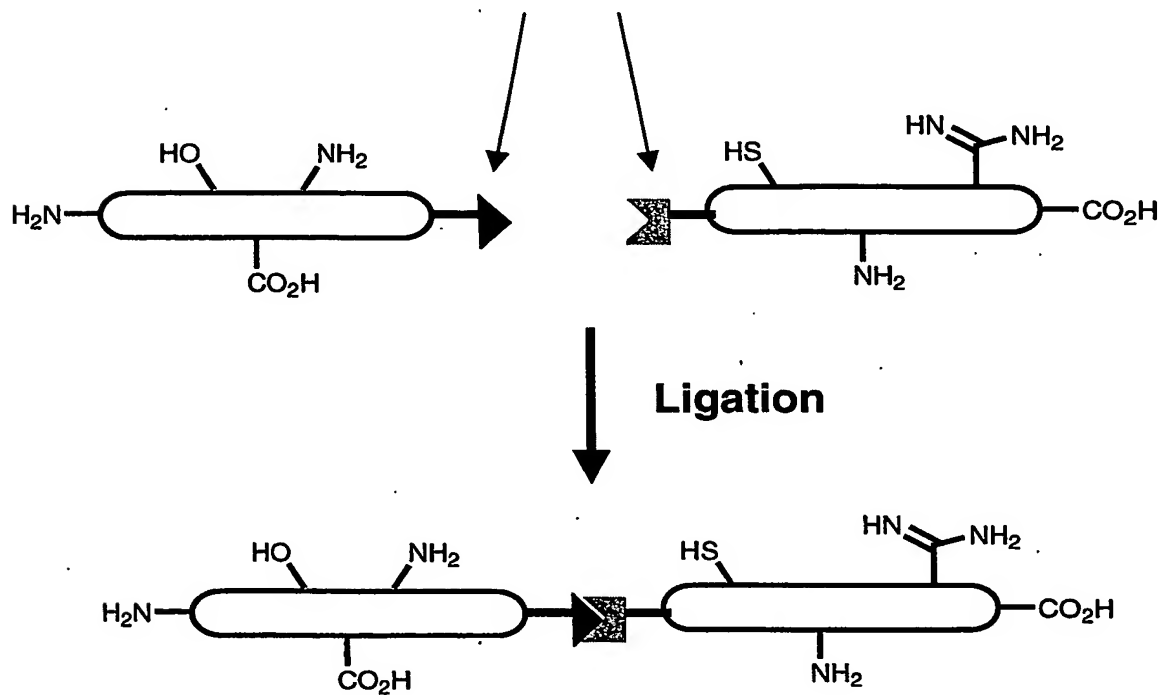


Figure 1 General principle of chemical ligation.



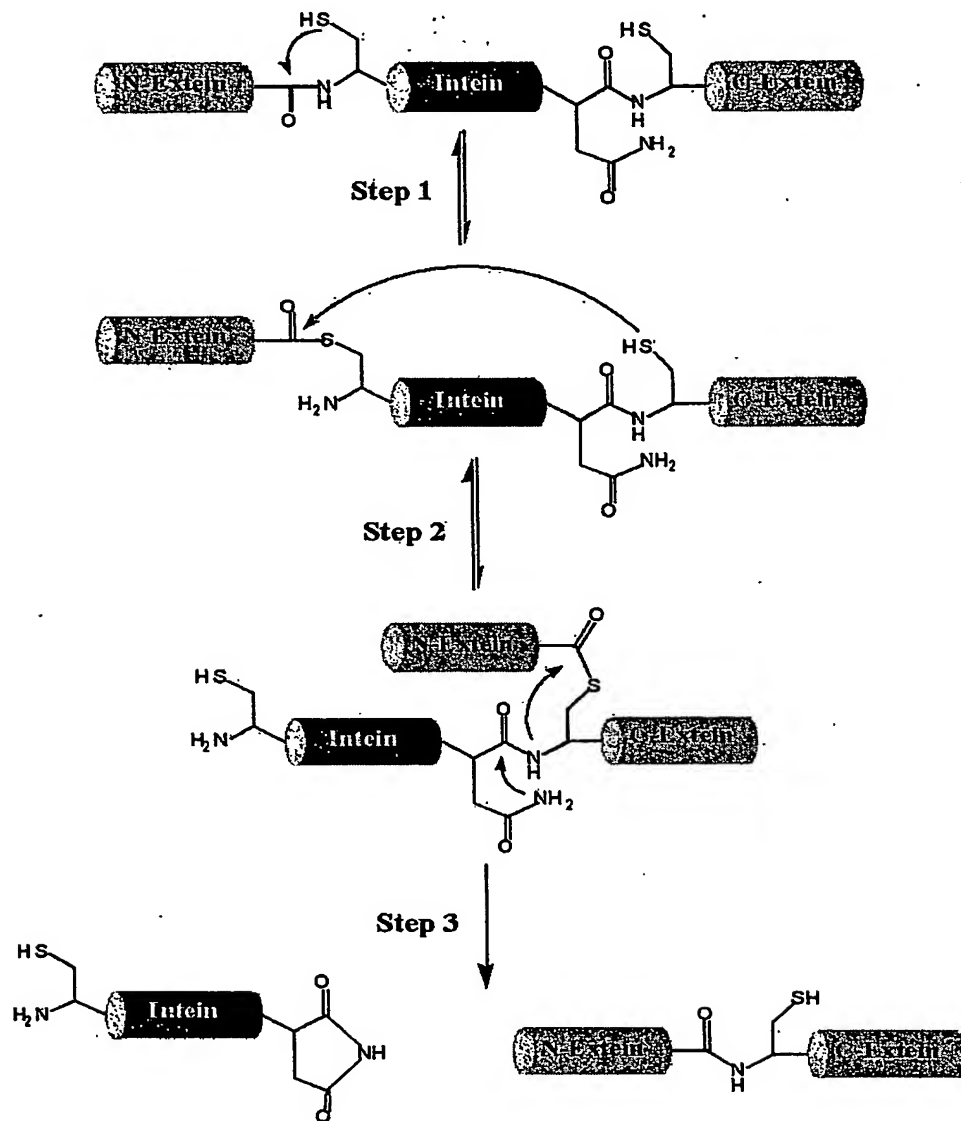
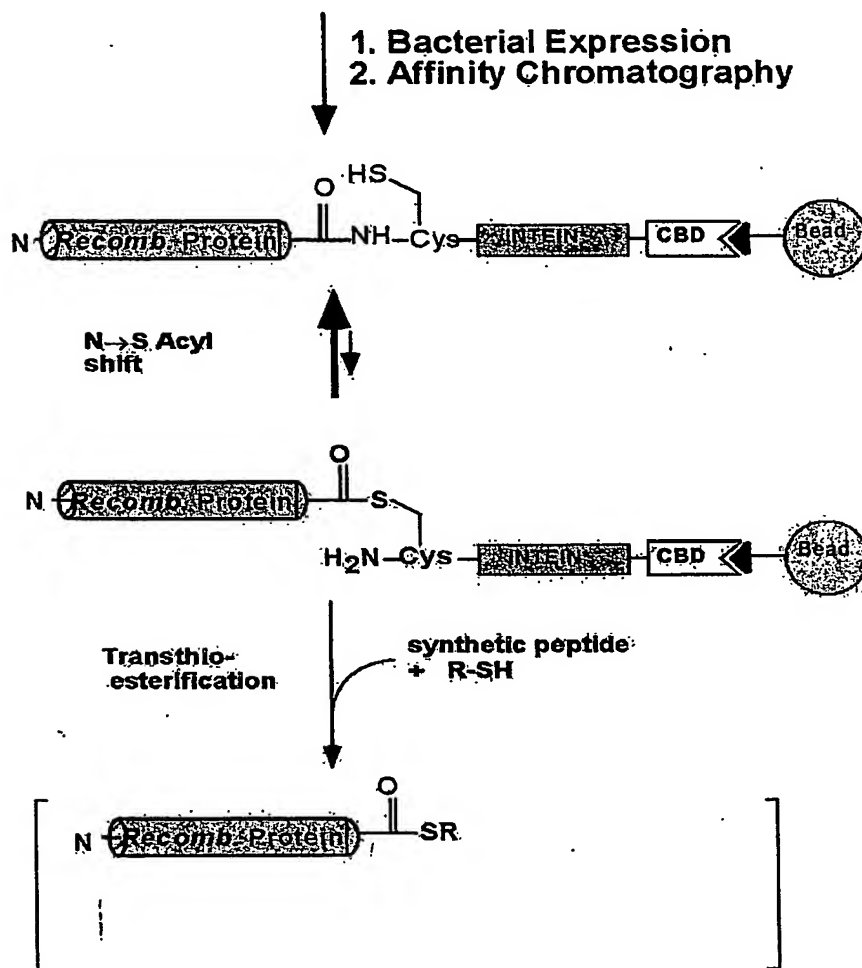


Figure 2 Mechanism of protein splicing

**Clone Gene into Engineered  
Intein Expression Vector**



**Figure 3 Generation of Recombinant C-terminal Thioester Proteins**

Synthetic or recombinant peptide / protein -thioester

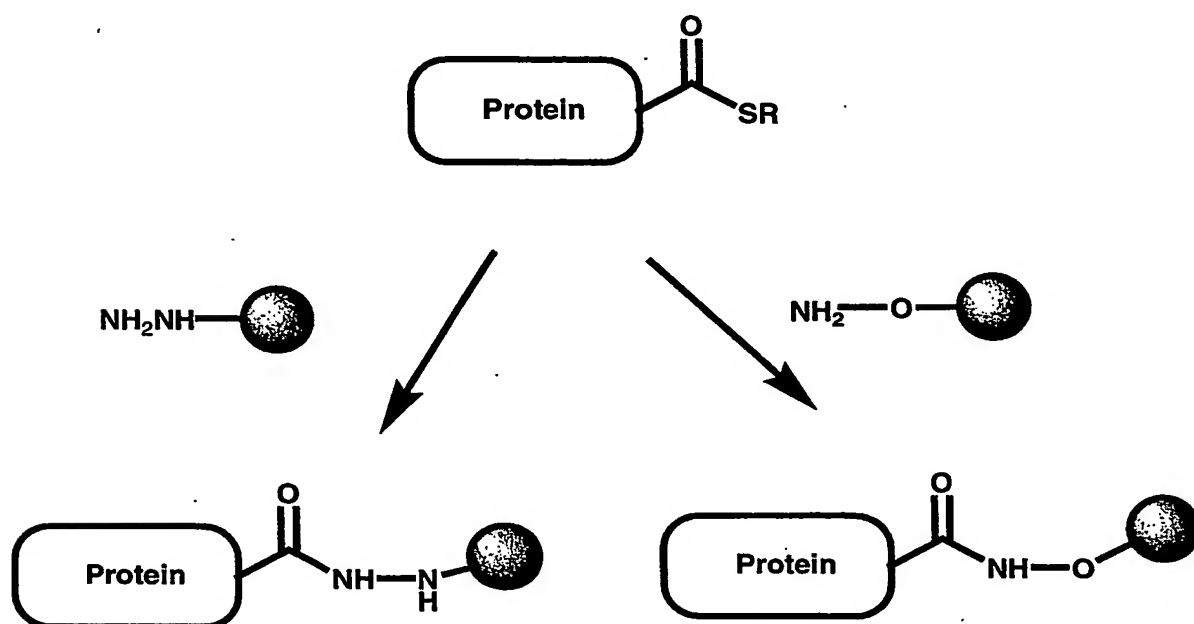


Figure 4 Ligation of protein and peptide thioesters with hydrazine and aminooxy containing entities such as labels, peptides and proteins.

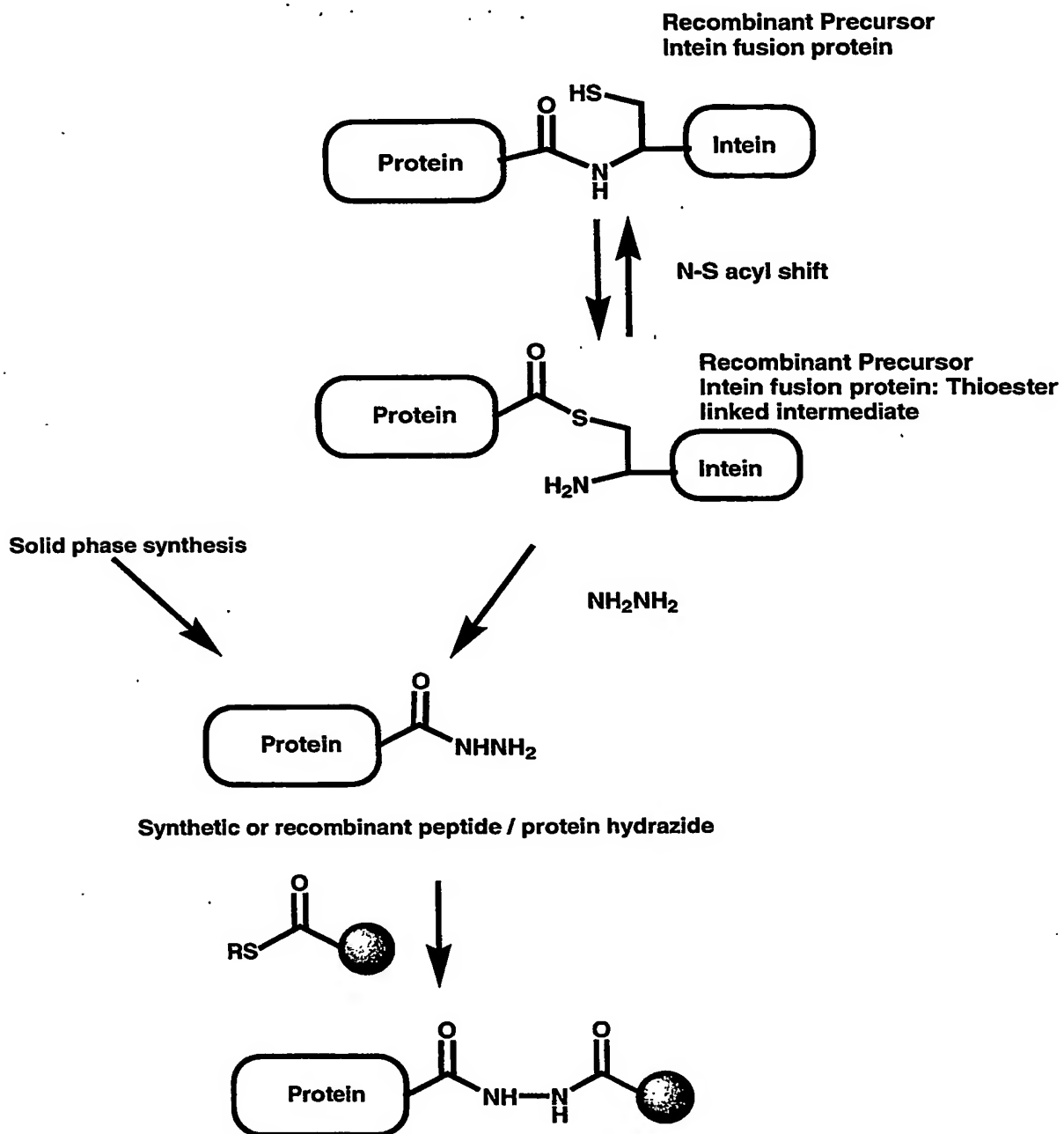


Figure 5 Generation of synthetic and recombinant peptide hydrazides for ligation with thioester containing molecules

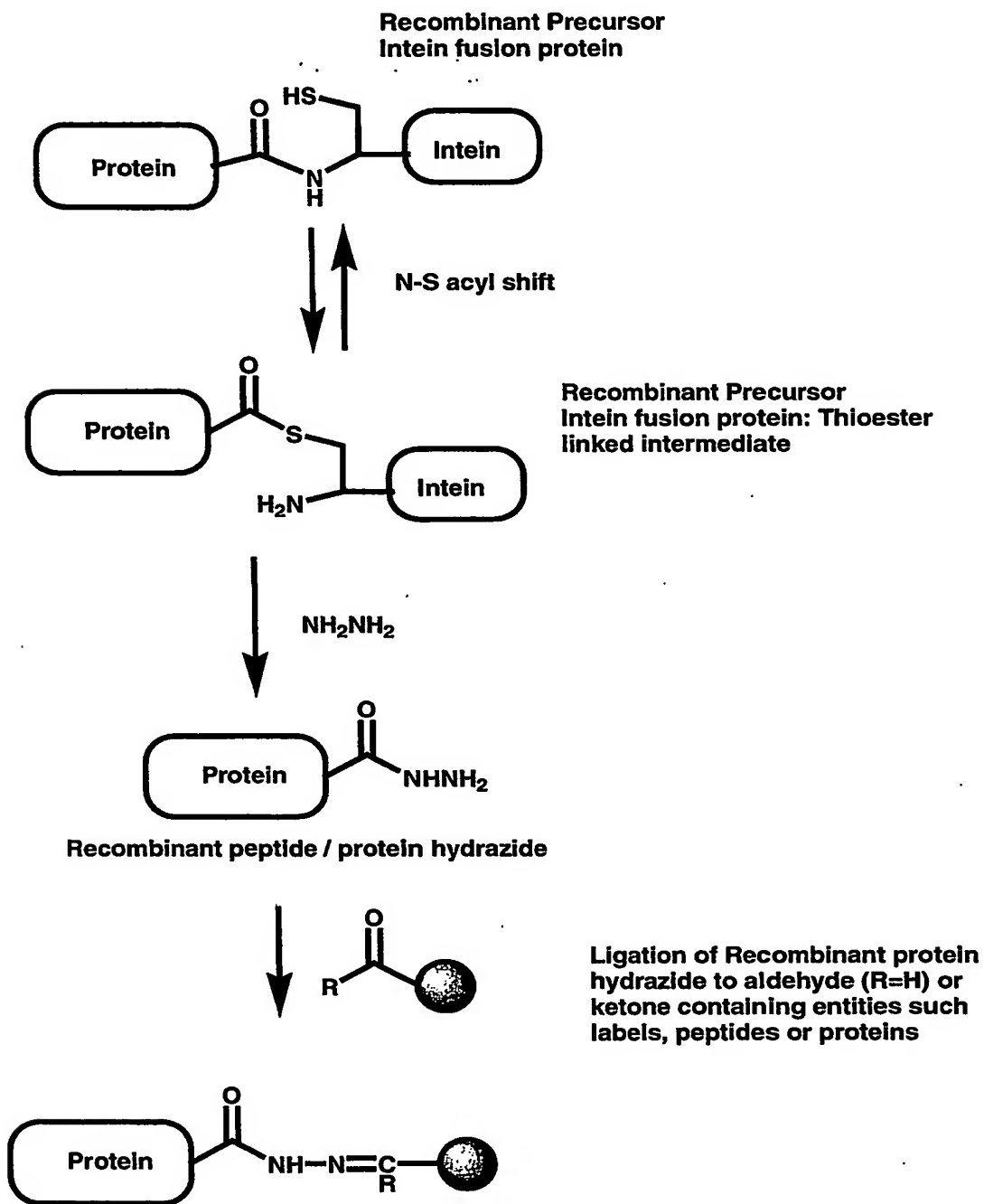


Figure 6 Generation of recombinant peptide hydrazides for ligation with aldehyde and ketone containing molecules

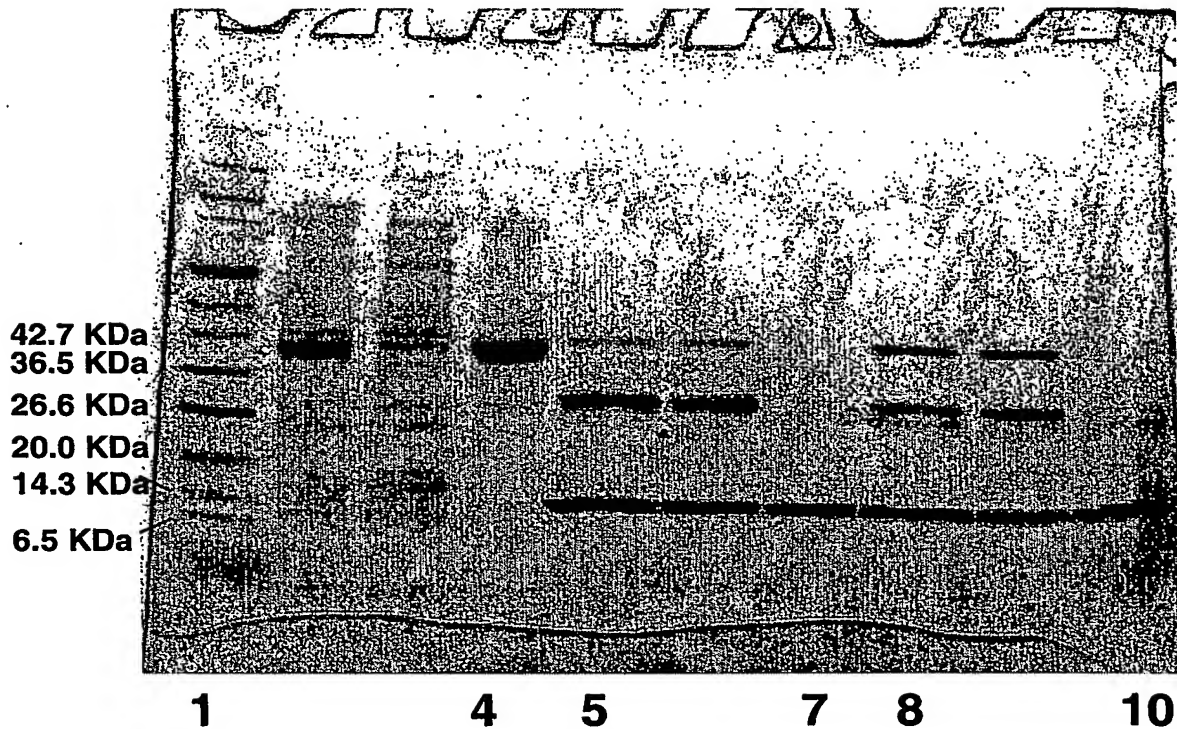


Figure 7. SDS-PAGE analysis of Grb2-SH2 – GyrA – CBD (immobilised on chitin beads) treated with DTT and MESNA. Molecular weight markers (lane 1); purified Grb2-SH2 – GyrA – CBD immobilised on chitin beads (lane 4). Grb2-SH2 – GyrA – CBD treated with 100 mM DTT (lanes 5 and 7) or 120 mM MESNA (lanes 8 and 10). Both the whole reaction slurries (lanes 5 and 8) and the reaction supernatants (lanes 7 and 10) were analysed.

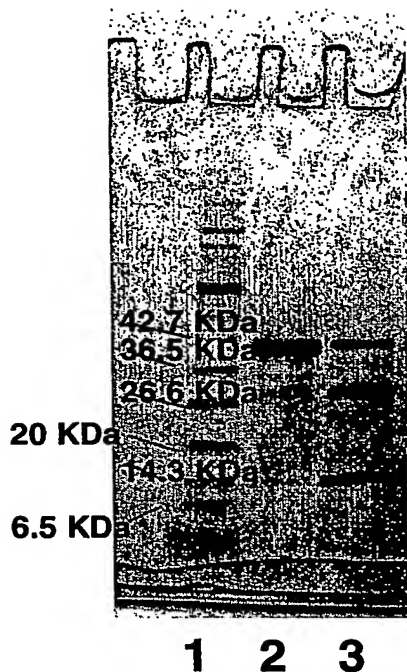


Figure 8. SDS-PAGE analysis of Grb2-SH2 – GyrA – CBD (immobilised on chitin beads) treated with hydrazine. Molecular weight markers (lane 1); Purified Grb2-SH2 – GyrA – CBD immobilised on chitin beads after 20h treatment with phosphate buffer only (lane 2). Grb2-SH2 – GyrA – CBD treated with 200 mM hydrazine in phosphate buffer for 20 h. The whole reaction slurries were analysed.

9/10

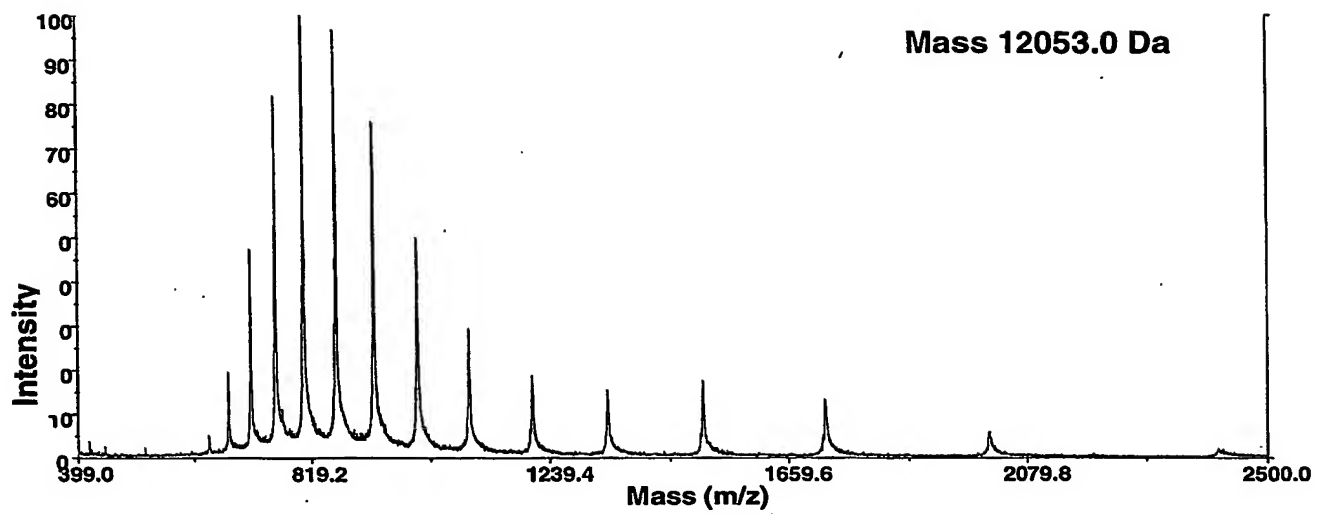


Figure 9. ESMS spectrum of the C-terminal hydrazide derivative of Grb2-SH2



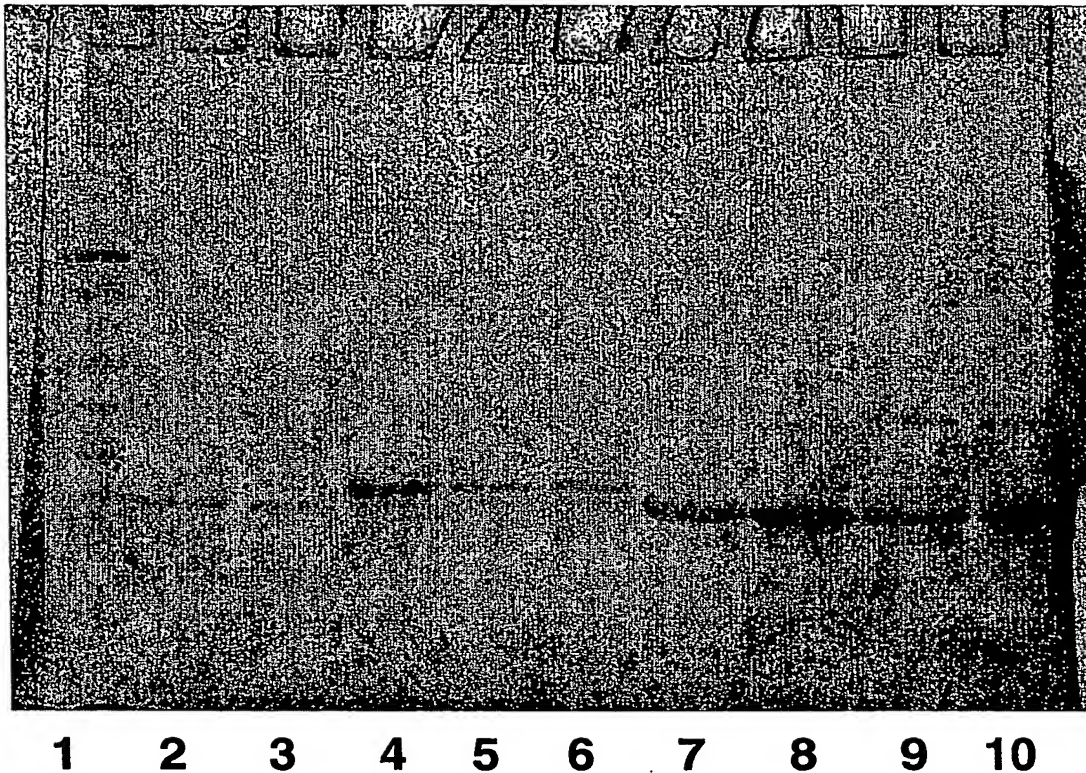


Figure 10. SDS-PAGE analysis of the reaction between synthetic ketone containing peptide CH<sub>3</sub>COCO-myc with Grb2-SH2 – C-terminal hydrazide and Cytochrome C. Molecular weight markers (lane 1); Grb2-SH2 – C-terminal DTT thioester (lane 2). Reaction between Grb2-SH2 – C-terminal hydrazide and CH<sub>3</sub>COCO-myc at time points t=0 h (lane 3), t=24 h (lane 4), t= 48h (lane 5) and t= 72 h (lanes 6). Reaction between Cytochrome C and CH<sub>3</sub>COCO-myc at time points t=0 h (lane 7), t=24 h (lane 8), t= 48h (lane 9) and t= 72 h (lanes 10).